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Pazzaglia G, Winoto I, Jennings G

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Naval Medical Research Institute
Commanding Officer
8901 Wisconsin Avenue
Bethesda, Maryland 20889-5607

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Oral Challenge with *romonas* in Protein-Malnour... Mice

G PAZZAGLIA, I WINOTO, AND G ... NINGS

U.S. Naval Medical Research Unit No. 2, Jakarta, Indonesia.

ABSTRACT

The lack of an animal model for *Aeromonas*-associated diarrhoea has hindered progress toward understanding the pathogenesis of this potentially important enteric infection. Protein-malnourished mice were challenged orally with *Aeromonas* strains to determine if diminished levels of resistance would allow the induction of a diarrhoeal response. The 15 *Aeromonas* spp. faecal isolates used for challenge included 7 *A. caviae*, 4 *A. hydrophila*, 1 *A. sobria* bv. *sobria*, and 1 *A. sobria* bv. *veronii* from patients with diarrhoea, and 2 *A. caviae* from healthy volunteers. All had at least 1 known virulence marker, with the exception of 1 strain. Mice on the protein deficient diet had lost an average of 23% of their initial body weight at the time of challenge. Although mice consumed 10^8 cfu per day for a minimum of 4 days, none became ill due to *Aeromonas* spp. ingestion. *Aeromonas* spp. were isolated from 75% of faecal cultures obtained 7 days after initial challenge, indicating bowel colonization had occurred.

Key words: *Aeromonas*; Animal model; Malnutrition; Protein deficiency; Diarrhoea.

INTRODUCTION

The more frequent faecal isolation of *Aeromonas* spp. from patients with diarrhoea compared to healthy individuals (1-5) and the large proportion of *Aeromonas* strains that produce numerous potential virulence factors (6,7) supports the conviction by many that some phenotypes of *Aeromonas* are important enteropathogens. However, several case-control studies (8-11) and community-based longitudinal studies (12-14) have shown a weak or non-existent association with disease. Furthermore, challenges in adult humans failed to produce disease (15), and there has been no outbreak unequivocally linked to an *Aeromonas* strain. A proven virulence marker for diarrhoeagenic strains of *Aeromonas* spp. has not been identified.

An animal diarrhoea model of *Aeromonas* spp. infection would help clarify these apparent contradictions regarding this organism's role in human infectious diarrhoeas and provide an understanding of the pathogenesis of *Aeromonas*-associated disease. To date, no model has been reported which produces diarrhoea in *Aeromonas*-challenged animals *in vivo*.

The highest prevalence of *Aeromonas*-associated diarrhoea and the strongest association with disease have been reported for studies performed in developing countries such as Bangladesh (16) and Peru (5), where children hospitalized with diarrhoea are frequently suffering from malnutrition, especially due to protein-deficient diets (17). Protein and other nutritional deficiencies have been shown to have adverse effects on the host's ability to mount a successful defense following infection by common viral and bacterial agents. Among those defenses shown to be diminished by malnutrition are mucosal barriers, serum and secretory antibody responses, and the cell-mediated immune response (17).

We hypothesized that a diminished level of resistance in malnourished mice would allow the induction of a diarrhoeal response when challenged with *Aeromonas* strains. Our objective was to test selected strains of *Aeromonas* for their ability to produce diarrhoea in mice malnourished by a protein-deficient diet.

MATERIALS AND METHODS

Experimental design. Four identical experiments were performed, each using 4 strains of *Aeromonas* spp. for challenge (15 strains total). For each experiment, mice were allocated into 4 challenge groups and 2 weight-control groups. Groups consisted of 4 to 6 mice each. The 4 challenge groups were mice which had been fed a protein-deficient diet for 7 days, and then challenged by placing *Aeromonas*-inoculated water in their water bottles (Day 0). The 2 weight-control groups served as

Correspondence and reprint requests should be addressed to:
Dr. G Pazzaglia, Naval Medical Research Institute
8901 Wisconsin Avenue, Bethesda, Maryland 20889-5607, U.S.A.

controls for measuring the severity of malnourishment of mice while on the protein-deficient diet. One weight-control group was provided a complete diet, and the other was given the same protein-deficient diet as the challenge groups. There was no normal diet challenge group. Challenged animals were observed for 7 days after switching their drinking water to *Aeromonas* strain suspensions. All challenge groups consumed viable bacterial suspensions for at least 4 days.

Animal use. The housing, treatment, and experimental use of animals complied with the Guidelines of the Committee on Care and Use of Laboratory Animals of the National Research Council.

Source of challenge strains. All of the challenge strains were from previously reported population studies (5,14). Thirteen of the 15 challenge *Aeromonas* spp. faecal isolates were from Peruvian children with diarrhoea who were less than 2 years of age. No other enteropathogens were detected in their stools. All of the 10 children had a secretory type diarrhoea, 5 had mucus in their stools (strain no. 1, 3, 4, 11, 14), 6 had associated fevers (strain no. 1, 4, 6, 10, 11, 14), but none had bloody stools. Two of the challenge strains were from healthy control children (strain no. 7, 12).

Challenge strain phenotypes. Because no individual strain, biochemical phenotype, or virulence marker phenotype of *Aeromonas* spp. has been proven to be diarrhoeagenic in animals or humans, challenge strains were selected which represented a variety of characteristics and phenotypic profiles (Table I), as determined previously (18). In total, there were 9 *A. caviae*, 4 *A. hydrophila*, 1 *A. sobria* bv. *sobria*, and 1 *A. sobria* bv. *veronii* (metabolically phenotyped by the method of Janda [6]); 10 strains were reactive in more than 1 cell culture type, 4 were haemolytic, 3 spontaneously pelleted in broth (SP+ [7]), 2 precipitated after boiling (PAB+ [7]), 3 were positive in the infant mouse assay for enterotoxin (19,20), 3 were positive in the rabbit ileal loop assay for enterotoxin (21,22), and 6 were lethal to mice when injected intraperitoneally with at least 10^7 organisms.

Preparation of challenge inoculum. For preparation of each challenge inoculum, 3 to 5 *Aeromonas* colonies were picked from fresh growth on MacConkey agar and suspended in 50 ml brain heart infusion broth. Broth cultures were incubated 18 h at 36+1°C in a shaking incubator (200 rpm). Overnight cultures were centrifuged (2,000 x g for 30 min at 5°C), the supernatant decanted, and the pellets resuspended in 50 ml sterile phosphate buffered saline (PBS) by vortexing. Bacterial suspensions were again centrifuged and decanted, and the pellet was resuspended in 200 ml sterile unchlorinated water. These suspensions served as the challenge inocula and were placed in individual feeding bottles which had been rinsed with 70% ETOH and dried before filling.

At the time challenge suspensions were prepared, each suspension was titrated by serially diluting 1.0 ml and inoculating dilutions onto MacConkey agar plates. Colony-forming units (cfu) per ml were calculated after counting *Aeromonas* spp. colonies (lactose-negative colonies; typical morphology) on each plate following overnight incubation at 36±1°C. Titrations were also performed on challenge suspensions sampled on days 1, 2, and 5, although not all suspensions were titrated on every occasion.

Challenge procedure. Following weaning at 24 to 25 days of age, C3H mice were placed on a diet of commercially bottled water and formulated food. Two mice from each litter (normal diet weight control-group) received a complete diet (Normal Protein Diet for Rat or Mouse, ICN Biomedicals, Cleveland, OH), and the remaining mice of each litter (challenge groups + malnourished weight control group) received a protein-deficient diet (Protein Free Diet, ICN Biomedicals; a semi-synthetic rodent food composed of 67% sucrose, 15% corn starch, 8% non-nutritive bulk, 5% corn oil, and 5% vitamin and mineral supplements). Weight measurements (pooled) were made at least every 2 days. When the protein-malnourished mice demonstrated a >20% pooled weight loss compared to their initial body weight (day 7), the diet deficient mice were allocated into separately caged groups (4 to 6 mice of the same sex in each group) for challenge with *Aeromonas* strains, or continued observation as an unchallenged weight-control group. At the time of challenge, bedding was removed and the floor of the cage was left bare (absorbent paper was not used because the animals shredded the material overnight, even when it was firmly taped to the cage bottom). Animals and the cage bottom were observed daily for signs of diarrhoea (faecal material on the anal-rectal area, or signs of liquid stool on the cage bottom, respectively). The animals were placed into clean cages each day, with special precautions taken to prevent cross-contamination. When challenge suspensions were depleted (usually on day 4 or 5), drinking bottles were replaced with containers of their regular water.

Challenge dosages. Serial dilutions of samples from challenge inocula yield 9.3 x 10^7 to 6.0 x 10^9 cfu per ml, with the average inoculum being 8.9 x 10^8 cfu per ml. As mice consumed approximately 10 ml of the bacterial suspension per day per mouse, the average challenge dose for each mouse on the first day of challenge (day 0) was approximately 9.0 x 10^9 cfu. All challenge groups consumed the bacterial suspensions for at least 4 days. Die-off of *Aeromonas* spp. organisms in the drinking water was slow; by days 1, 2, and 5 post-challenge, *Aeromonas* spp. average log viable counts had declined by 0.40, 0.62, and 1.00, respectively. This indicated the mice were being continuously challenged through day 4.

Faecal cultures. For determinations of mouse colonization by *Aeromonas* strains, faeces were obtained from the bottoms of cages on days 1, 2, 5, 6, and 7. Not all groups were cultured on all days. Faeces were vortexed in 1 ml of sterile PBS, then 0.01 ml of the suspension was directly inoculated onto MacConkey agar and incubated overnight at 36±1°C. To enrich for *Aeromonas* spp., 10 ml of alkaline peptone water was

added to the remaining suspension, incubated overnight at ambient temperature, then subcultured onto MacConkey agar and incubated overnight at $36 \pm 1\,^{\circ}C$. Direct cultures and enriched subcultures were inspected for suspect *Aeromonas* spp. colonies. Lactose-negative, oxidase-positive colonies were confirmed *Aeromonas* spp. using conventional biochemical tests (24).

Table I. Phenotypic characteristics of 15 *Aeromonas* strains used for oral challenge of protein-malnourished mice.

Strain number	*Aeromonas* species	Agglutination[a,b] SP	PAB	Cytotoxin[a,c] SRBC	Y1	CHO	Vero	Enterotoxin[a,d] mouse	rabbit	Invasiveness mouse[a,e]
1	hydrophila	-	-	+	+	+	+	-	-	nt[a]
2	hydrophila	-	-	+	+	+	+	-	-	+
3	hydrophila	-	-	+	+	+	+	-	-	+
4	hydrophila	-	+	-	-	+	+	-	-	nt
5	sobria bv. sobria	-	-	+	+	+	+	+	-	+
6	sobria bv. veronii	nt	nt	-	+	+	+	-	-	-
7	caviae	-	-	-	-	+	+	+	+	+
8	caviae	-	-	-	+	+	+	-	+	nt
9	caviae	-	-	-	+	+	+	-	-	+
10	caviae	+	-	-	-	-	+	+	-	+
11	caviae	+	+	-	-	-	-	-	+	+
12	caviae	-	-	-	+	-	+	-	-	-
13	caviae	-	-	-	-	+	-	-	-	-
14	caviae	-	-	-	-	-	-	-	-	+
15	caviae	-	-	-	-	-	-	-	-	-

[a] *Abbreviations*: SP, spontaneous pelleting; PAB, precipitation after boiling; SRBC, sheep red blood cells (hemolysis); Y1, adrenal cell culture; CHO, Chinese hamster ovary cell culture; Vero, monkey kidney cell culture; nt, not tested.

[b] Agglutination phenotypes as described by Janda (7)

[c] Causes cytotonic or lytic response.

[d] Causes intestinal fluid accumulation.

[e] Lethal if injected intraperitoneally at a dose of 10^7 cfu (23).

RESULTS

Effects of protein-deficient diet. The figure illustrates the average weight loss in 23 control mice fed a 0% protein diet compared to the weight gains of 21 mice fed a complete diet (combined results of 4 experiments). After 7 days, unchallenged control mice fed the protein-free food had lost an average 23% of their original body weight, whereas unchallenged mice fed a complete diet had gained an average 35.9% of their original body weight. By day 18, mice fed the protein-free diet had lost 31.6% of their body weight, and normal-diet mice had gained 106.1%.

Colonization. Ten of the 15 (75.0%) challenged groups yielded *Aeromonas*-positive faecal specimens, even though some groups were only cultured once (data not shown). *Aeromonas*-positive faeces from the bottoms of cages demonstrated that *Aeromonas* strains are able to transit the gastric-acid barrier without the use of a feeding tube or buffered liquid vehicle. The majority of mice cultured were still shedding the organism 1 to 3 days after the *Aeromonas* challenge suspensions had been replaced (Table II).

Virulence of challenge strains. Four separate challenge experiments were performed, using 4 *Aeromonas* strains in each experiment (1 strain was repeated). There were no illnesses or deaths in challenged mice which could be attributed to *Aeromonas* challenge strains.

In one experiment, 3 of 4 mice challenged with strain no. 14 became ruffled and lethargic on day 4, but showed no signs of diarrhoea. Two moribund mice in this group were sacrificed and a necropsy revealed no fluid accumulation in the bowel or other abnormalities. Samples of heart blood were cultured and found to be sterile. The 2 surviving mice died on day 6. A necropsy on 1 of these mice again showed no abnormalities and a culture of post-mortem heart blood was sterile. Strain no. 14 was retested in a later experiment; all animals survived with no evidence of illness.

DISCUSSION

Although several animal assays *in vivo* have been used to identify *Aeromonas* strains with potential virulence markers (6), there have been no reports of successfully inducing diarrhoea through oral challenges of animals not surgically manipulated. Challenges using the removable intestinal tie adult rabbit diarrhoea (RITARD) model (25) have demonstrated some strains to be highly virulent, but only after invasion of the gut mucosa, bacteraemia, and death due to sepsis (26). In the same investigation, RITARD challenges of rabbits with invasive strains of *Aeromonas* were shown to infrequently produce diarrhoea. When diarrhoea did occur, it was not voluminous and contributed little to the outcome of the challenged rabbits.

The current investigation, using a mouse protein deficiency model, provided no evidence that a murine host can be made more susceptible to *Aeromonas* spp. infection through a dietary-induced state of malnutrition. The deaths in mice which had been challenged with strain no. 14 were considered to have resulted from an unrelated cause. Necropsies of moribund mice failed to demonstrate evidence of infection from the *Aeromonas* challenge strain, and a second experiment, using the same strain, failed to produce illness in challenged mice. Considering that all of the mice that became ill were housed in a single cage, we hypothesize that undetected microenvironmental changes may have led to the diminished physiological and health status of these animals.

Although infant mice (3 to 5 days of age) have previously been used for detection of *Aeromonas* spp. enterotoxin (13,20), we looked for a diarrhoeic response in older mice (24 to 25 days). Weanling mice were used because the day of weaning is the earliest age at which mice can easily be placed on a protein-free diet. It is possible that some of the orally fed strains of *Aeromonas* spp. might have caused diarrhoea had they been fed to

younger protein-malnourished mice, but a protein-deficient substitute for colostrum was not available.

The challenge experiments were unsuccessful in inducing *Aeromonas*-associated diarrhoea using a protein-malnourished mouse model. However, there were several observations which may prove beneficial to diarrhoeal disease researchers. First, it is possible to at least temporarily colonize mice with *Aeromonas* strains using oral inocula. Asymptomatic carriage of *Aeromonas* strains in humans is common in some developing countries (5,10,27), and transient colonization in challenged rabbits has been previously reported (26). Although some species of laboratory animals have been found to be naturally colonized, others have not (28). A mouse-colonization model may be useful in determining what factors lead to intestinal carriage in some human and animal populations, but not others.

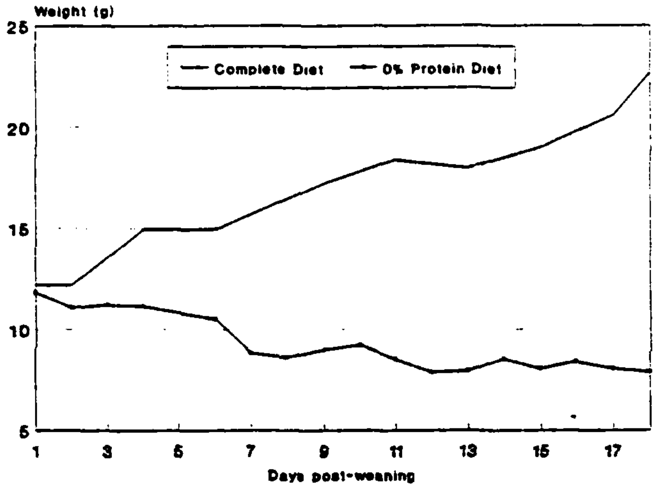

Figure. Average individual weight loss of mice fed a 0% protein diet (n = 23) compared to the weight gains of mice (n = 21) fed a complete diet at the time of weaning; combined results of 4 experiments.

Aeromonas spp. are known to be aquatic organisms (29) and have been shown to survive as long as 2 weeks on a moist surface in a humid environment (28). As expected, the challenge strains were capable of surviving for relatively long periods in drinking water with no added nutrients. This fact, considered with the observation that the challenge organisms were able to transit the gastric acid barrier without the use of a buffered vehicle, supports the notion that consumption of contaminated water is an important source of human enteric colonizations by *Aeromonas* spp. Modified versions of this challenge procedure may prove useful to researchers for investigations of strain-specific colonization factors, cell adherence, or other interactions between the organism and the intestinal epithelium.

Lastly, a protein-deficient diet rapidly and drastically interferes with normal growth of weanling mice, although the immunological effects of protein starvation were not measured in this series of experiments. The malnourished state did not seem to affect the susceptibility of mice to *Aeromonas* spp. infection, but there may be other enteropathogens which could be used in similar models to study the interaction of malnutrition and diarrhoeal disease in humans.

Table II. Colonization of *Aeromonas* strains in groups of orally challenged mice (4 to 6 mice per group); mice were fed *Aeromonas* strain suspensions in drinking water on days 1 through 5.

	Day of experiment					
	1	2	5	6	7	Overall
N groups cultured	8	8	4	4	4	28
N groups Aeromonas-positive	3	6	2	2	3	16
% groups Aeromonas-positive	37.5	75.0	50.0	50.0	75.0	57.1

The current study was planned as the first in a series of protein-deficiency experiments, and so did not include challenged normal diet control groups. The determination that none of the 15 *Aeromonas* strains tested caused diarrhoea in protein-malnourished mice precluded the expenditure of additional mice to test other strains, or for use as additional controls. Future investigations using murine models for studies of other enteropathogens in malnutrition-associated diarrhoea will require the use of such controls.

The lack of an animal model for *Aeromonas*-associated diarrhoea continues to hinder progress in studies of pathogenic mechanisms for this newly recognized and potentially important enteropathogen. Despite the current study's outcome, it is likely that future investigations using murine models will contribute toward a better understanding of the pathogenesis of *Aeromonas*-associated diarrhoea in humans, and an improved ability to distinguish virulent strains of these organisms.

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